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Reactive Oxygen Species prime *Drosophila* haematopoietic progenitors for differentiation

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Abstract

Reactive Oxygen Species (ROS), produced during various electron transfer reactions *in vivo* are generally considered to be deleterious to cells1. In the mammalian haematopoietic system, haematopoietic stem cells (HSCs) contain low ROS levels, but unexpectedly, the common myeloid progenitors (CMPs), produce significantly elevated levels of ROS2. The functional significance of this difference in ROS level in the two progenitor types remains unresolved2,3. Here, we show that *Drosophila* multipotent haematopoietic progenitors which are largely akin to the mammalian myeloid progenitors4 display elevated levels of ROS under *in vivo* physiological conditions, which is downregulated upon differentiation. Scavenging the ROS from these haematopoietic progenitors using *in vivo* genetic tools, retards their differentiation into mature blood cells. Conversely, increasing the haematopoietic progenitor ROS beyond their basal level triggers precocious differentiation into all three mature blood cell types found in *Drosophila*, through a signaling pathway that involves JNK and FoxO activation as well as Polycomb downregulation. We conclude that the developmentally regulated, moderately high ROS level in the progenitor population sensitizes them to differentiation, and establishes a signaling role for ROS in the regulation of haematopoietic cell fate. Our results lead to a model that could be extended to reveal a probable signaling role for ROS in the differentiation of CMPs in mammalian haematopoietic development and oxidative stress response.

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Full methods accompany this paper.

Supplementary information is linked to the online version of the paper at www.nature.com/nature.

Author Contributions UB supervised the project. EOA conceived, designed and performed all experiments. EOA and UB discussed results and wrote the manuscript.

Keywords

Drosophila; blood; ROS; JNK; FoxO; Haematopoiesis

The *Drosophila* lymph gland is a specialized haematopoietic organ which produces three blood cell types – plasmatocytes, crystal cells and lamellocytes – with functions reminiscent of the vertebrate myeloid lineage5,6. During the first and early second larval instars, the lymph gland is comprised essentially of only the progenitor population (Fig. 1a, lower panel). However, by late third instar, multipotent stem-like progenitor cells become restricted to the medial region of the primary lymph gland lobe, in an area referred to as the Medullary Zone (MZ); while a peripheral zone, referred to as the Cortical Zone (CZ) contains differentiated blood cells. By late third instar, the progenitors within the MZ are essentially quiescent, while the mature, differentiated population in the CZ proliferates extensively5. The Posterior Signaling Center (PSC), is a group of about 30 cells (Fig. 1a, upper panel), that secretes multiple signaling molecules7–9 and serves as a stem cell niche regulating the balance between cells that maintain "stemness" and those that differentiate8,9.

Although several studies have identified factors that regulate the differentiation and maintenance of *Drosophila* blood cells and the stem-like progenitor population that generates them8–11, intrinsic factors within the stem-like progenitors are less explored, and forms the central theme of this investigation. We observed that by the third instar, the progenitor population in the normal wild-type lymph gland MZ contain significantly elevated ROS levels when compared to their neighboring differentiated progeny that express mature blood cell markers in the CZ (Fig. 1b–e). ROS is not elevated during the earlier larval instars but rises as the progenitor cells become quiescent and subside as they differentiate (Fig. 1b–e). This first suggested to us that the rise in ROS primes the relatively quiescent stem-like progenitor cells for differentiation. We reduced ROS by expressing antioxidant scavenger proteins GTPx-112 (Fig. 1f,g) or Catalase (Supplementary Fig. 1), specifically in the progenitor cell compartment using the GAL4/UAS system13, and found that suppressing elevated ROS levels in haematopoietic progenitors significantly retards their differentiation into plasmatocytes (Fig. 1f,g and supplementary Fig. 1). As a corollary, mutating the gene encoding the antioxidant scavenger protein Superoxide Dismutase (sod2)1 led to a significant increase in differentiated cells and decrease in progenitors (Fig. 1h).

ROS levels in cells can be increased by the genetic disruption of complex I proteins of the mitochondrial electron transport chain14,15, such as ND75 and ND42 (Supplementary Fig. 2). Unlike in wild type, where early second instar lymph glands are exclusively comprised of undifferentiated cells (Fig. 2a), mitochondrial complex I depletion triggers premature differentiation of the progenitor population (Fig. 2b). This defect is even more evident in the third instar (Compare Fig. 2c and 2d), where a complete depletion of the progenitors is seen as primary lobes are populated with differentiated plasmatocytes and crystal cells. The third differentiated cell type, lamellocyte, defined by the expression of the antigen L1, is rarely observed in the wild-type lymph gland (Supplementary Fig. 3) but is abundantly seen in the mutant (Fig. 2e). Finally, the secondary and tertiary lobes, largely undifferentiated in wild

type, also embark on a robust program of differentiation upon complex I depletion (Fig. 2d, e and Supplementary Fig. 4). Importantly, the phenotype resulting from ND75 disruption can be suppressed by the co-expression of the ROS scavenger protein GTPx-1 (Fig. 2f, g; compare with Fig. 2d, e) providing a causal link between increased ROS and the premature differentiation phenotype. Combining these results with those in figure 1, we conclude that the normally elevated ROS levels in the stem-like progenitors serves as an intrinsic factor that sensitizes them to differentiation into all three mature cell types. Any additional increase or decrease in the level of ROS away from the wild-type level enhances or suppresses differentiation respectively.

In unrelated systems, elevated ROS levels have been demonstrated to activate the JNK signal transduction pathway1,16,17. Consequently, we tested whether the mechanism by which the progenitors in the MZ differentiate when ROS levels increase could involve this pathway. *puckered* (*puc*), is a downstream target of JNK signaling and its expression has been used extensively to monitor JNK activity18. Although *puc* transcripts are detectable by RT-PCR (Supplementary Fig. 5), the *puc-lacZ* reporter is very weakly expressed in wild-type (Fig. 3a). Upon disruption of ND75, however, a robust transcriptional upregulation of *puc-lacZ* expression can be seen (Fig. 3b), indicating that JNK signaling is induced in these cells in response to high ROS levels. The precocious progenitor cell differentiation caused by mitochondrial disruption is suppressed upon expressing a dominant negative version of Basket (Bsk), the sole *Drosophila* homologue of JNK (Fig. 3c, d; compare with Fig. 2d, e; also see Supplementary Fig. 5). This suppression was associated with a decrease in the level of expression (Supplementary Fig. 5) of the stress response gene encoding Phosphoenol pyruvate carboxykinase (PEPCK)19, and quantitatively, a 68% suppression of the ND75 crystal cell phenotype was observed when JNK function was removed as well (Fig 3e). Although disrupting JNK signaling suppressed differentiation, ROS levels remain elevated in the mutant cells (Supplementary Fig. 2f) as would be expected from JNK functioning downstream of ROS.

In multiple systems and organisms, JNK function can be mediated by activation of FoxO as well as through repression of Polycomb activity17,20,21. FoxO activation can be monitored *via* expression of its downstream target *thor*, using *thor-lacZ* as a transcriptional read-out22–24. *thor-lacZ* is undetectable in wild type lymph glands (Fig. 4a) although *thor* transcripts are detectable by RT-PCR (Supplementary Fig. 5), but the reporter is robustly induced when complex I is disrupted (Fig. 4b), suggesting that the complex I loss mediated increase in ROS activates FoxO. To monitor Polycomb derepression, we used a *Polycomb* reporter, which expresses *lacZ* when Polycomb proteins are downregulated. Although undetectable in wild-type lymph glands (Fig. 4c), disrupting ND75 leads to *lacZ* expression (Fig. 4d), suggesting that Polycomb activity is downregulated by the altered ROS and resulting JNK activation. Direct FoxO overexpression causes a remarkable advancement in differentiation to a time as early as the second instar (Fig. 4e), never seen in wild type (Fig. 2a). By early third instar, the entire primary and secondary lobes stained for plasmatocyte (Fig. 4f) and crystal cell (Fig. 4g) markers when FoxO is expressed in the progenitor population. Unlike with ROS increase, we did not find a significant increase in lamellocytes upon FoxO overexpression. However, downregulating the expression of two polycomb proteins,

Polyhomeotic Proximal (Php-x) and Enhancer of Polycomb (E(Pc)) that function downstream of JNK21 dramatically increased lamellocyte number (Fig. 4h) without affecting plasmatocytes and crystal cells (not shown). When FoxO and an RNAi against E(Pc) are expressed together in the progenitor cell population, differentiation to all three cell types is evident (Fig. 4i, j). We conclude that FoxO activation and Polycomb downregulation act combinatorially downstream of JNK to trigger the full differentiation phenotype – an increase in plasmatocytes and crystal cells due to FoxO activation, and an increase in lamellocytes primarily due to Polycomb downregulation.

The analysis of ROS in the wild-type lymph gland highlights a previously unappreciated role for ROS as an intrinsic factor that regulates differentiation of multipotent haematopoietic progenitors in *Drosophila*. Any further increase in ROS beyond the developmentally regulated levels, due to oxidative stress, will cause the progenitors to differentiate into one of three myeloid cell types. Tothova *et al.*2 reported that the ROS levels in mammalian HSCs is low but that in the CMPs is relatively high. The *Drosophila* haematopietic progenitors give rise entirely to a myeloid lineage and therefore, are functionally more similar to CMPs than they are to HSCs. It is therefore a remarkable example of conservation to find that they too have high ROS levels. The genetic analysis makes it clear that the high ROS in *Drosophila* haematopoietic progenitors primes them towards differentiation. It will be interesting to determine, if such a mechanism operates in mammalian CMPs. In mice, as in flies, a function of FoxO is to activate antioxidant scavenger proteins. Consequently, deletion of FoxO elevates ROS levels in the mouse HSC and drives myeloid differentiation2. However, even in the mouse haematopoietic system, FoxO function is dose and context dependent, as ROS levels in CMPs are independent of FoxO2. Thus, while the basic logic of elevated ROS in myeloid progenitors is conserved between flies and mice, the exact function of FoxO in this context may have diverged.

Our past work14 and those of others1,25,26 has hinted that ROS can function as signaling molecules at physiologically moderate levels. This work supports and further extends this notion. While excessive ROS is damaging to cells, developmentally-regulated ROS production, can be beneficial. The finding that ROS levels are moderately high in normal *Drosophila* haematopoietic progenitors and mammalian CMPs raises the possibility that wanton overdose of antioxidant products may infact inhibit formation of cells participating in innate immune response.

Methods Summary

Lymph glands were stained as previously described5,8 using the following antibodies: mouse anti-P1 and L1 (Ando, I.), rat anti-ProPO (Müller, H.), rabbit anti-βgal (Cappell) and mouse anti -βgal (Promega). Cy3, Cy5 and FITC conjugated secondary antibodies were from Jackson Laboratory. ROS staining was conducted as previously described14,15. Images were captured using a BioRad Radiance 2000 confocal microscope with LaserSharp 2000 acquisition software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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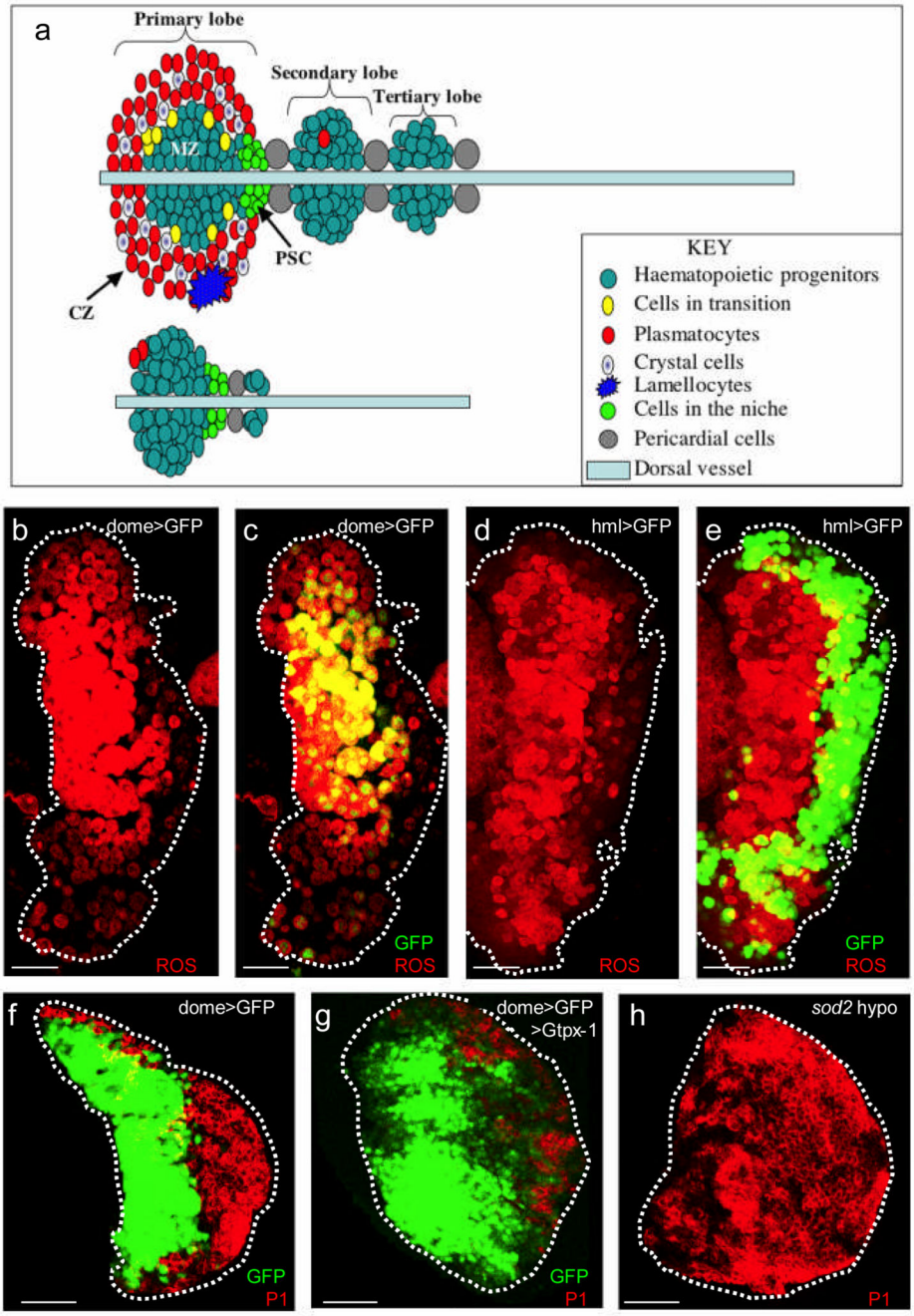

Figure 1. Reactive Oxygen Species Profile of third instar lymph glands

(a) Schematic diagrams of late third instar (upper panel) and early second instar (lower panel) lymph glands. The second instar lymph gland consists mostly of the progenitor population, which by late third instar, becomes restricted to the central domain of the primary lobe, referred to as the Medullary Zone (MZ). At least three differentiated cell types can be distinguished: plasmatocytes, crystal cells and lamellocytes. Lamellocytes are rarely found in wild-type lymph glands as they are only induced upon infection. All three differentiated cell types are largely restricted to the Cortical Zone (CZ). The third instar

lymph gland is comprised of several lobes; primary lobes are found in the most anterior region, and are followed posteriorly by two or more smaller lobes, referred to as secondary and tertiary lobes respectively.

(b) The progenitor population in the MZ show elevated ROS levels (red). The dotted outlines of lymph gland lobes in all panels are based on images acquired at high laser power.

(c) The expression of the MZ marker, *dome-gal4, UAS-2xEYFP* (green; genotype abbreviated on the panel as dome>GFP for clarity) overlaps with the ROS dye (red) in cells of the MZ (therefore yellow).

(d) As in panel (b), the progenitor population in the MZ show elevated ROS levels (red).

(e) *hml$^{\Delta}$-gal4, UAS-2xEGFP* is restricted to cells in the CZ (green). Most of the cells that are marked by *hml$^{\Delta}$-gal4, UAS-2xEGFP* are low in ROS (therefore green) when compared to cells in the MZ (red). A ring of *hml$^{\Delta}$-gal4, UAS-2xEGFP* expressing cells can be seen along the edge of the MZ that are both GFP and ROS positive (therefore yellow). These appear to be cells in a state of transition between the stem-like and the differentiated cell fate.

(f) Unlike in previous panels, the red color here marks P1 expression in differentiated plasmatocytes in the CZ. By late third instar, the expression of *dome-gal4, UAS-2xEYFP* (green) is restricted to the MZ and the cells in the CZ (red) downregulate this marker.

(g) Overexpression of the antioxidant protein (GTPx-1) in the progenitor cell compartment (genotype: *dome-gal4, UAS-2xEYFP; UAS-Gtpx1*) results in a pronounced reduction in the number of cells that express the P1 marker (red). Some cells occupying the CZ region continue to express *dome-gal4, UAS-2xEYFP* while many others downregulate this marker without yet expressing the differentiation marker P1.

(h) In the hypomorphic (weak allele) *sod2/sod2* homozygotes, in which the level of expression of a major ROS scavenger is reduced, P1 expression is expanded and can be found throughout the lymph gland (red), rather than being restricted to the CZ. This image is generated from the optical sections acquired from the central part of the gland.

Scale bars : 50μm.

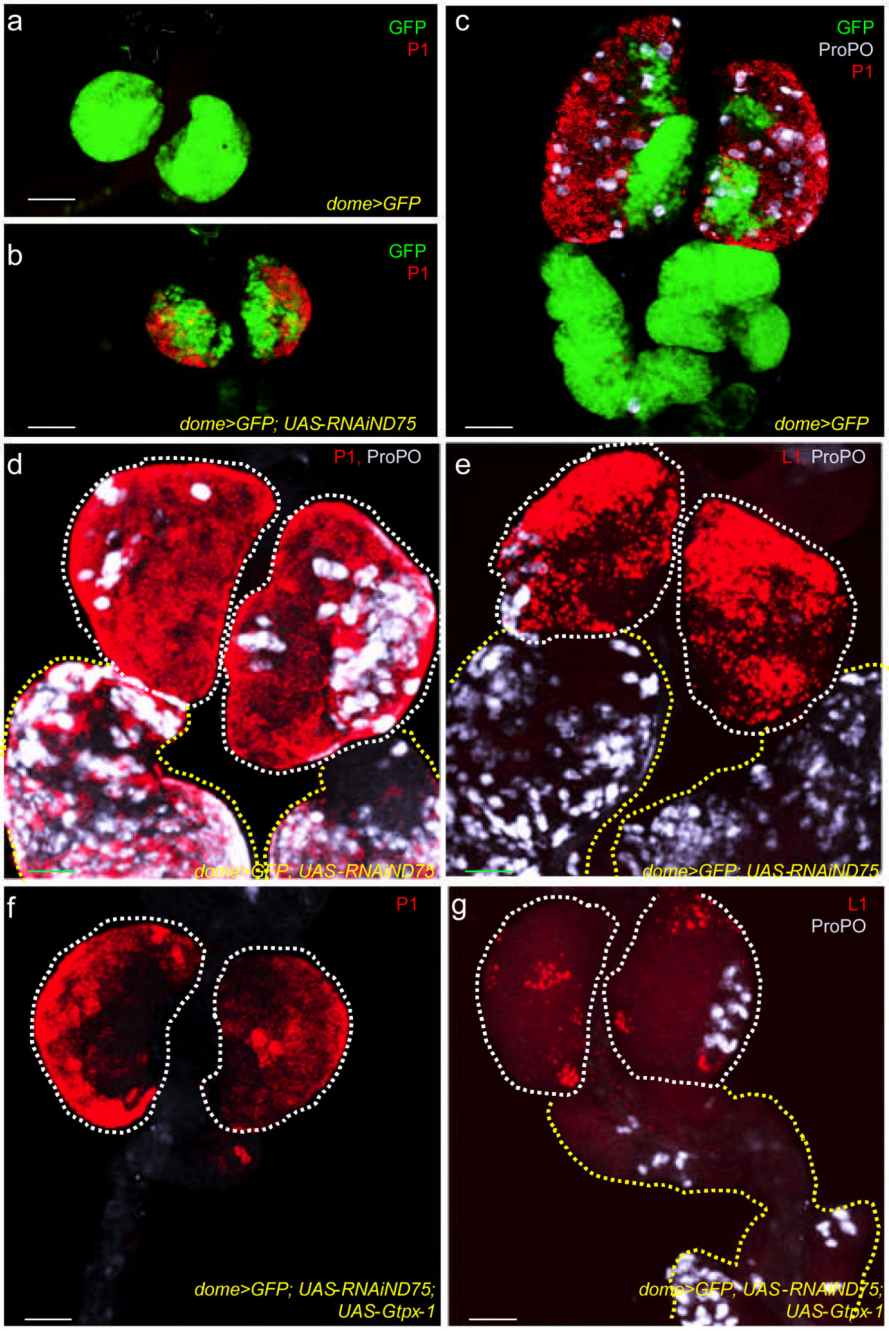

Figure 2. Increased ROS production triggers precocious differentiation of the multipotent progenitors

In all panels, the progenitor population expresses the MZ marker, *dome-gal4, UAS-2xEYFP* (green). In panels (d–g), the green channel has been omitted for clarity. The two genotypes used in these panels are control lymph glands (*dome-gal4, UAS-2xEYFP*), abbreviated as wild-type (WT), and experimental lymph glands which express a RNAi construct to *ND75* (*dome-gal4, UAS-2xEYFP; UAS-RNAiND75)*, abbreviated as $ND75^{RNAi}$. Scale bars :50μm. **(a)** P1 is not expressed (note absence of red) in early second instar WT lymph glands.

(b) P1 expression (red) is robustly induced in early second instar *ND75*^{RNAi} lymph glands.
(c–e) Disruption of ND75 triggers precocious differentiation.
(c) In WT third instar lymph glands, plasmatocytes marked with P1 (red) and crystal cells with ProPO (gray) are restricted to the CZ. These differentiated cell types are rarely if ever found in secondary and tertiary lobes.
(d) In third instar *ND75*^{RNAi} lymph glands, there is a dramatic increase in P1 (red) and ProPO (gray) expressing cells, throughout the primary, as well as in the secondary and tertiary lobes (tertiary lobes are shown in Supplementary Fig. 4).
(e) Lamellocytes, marked by L1 (red) are prominently seen in third instar *ND75*^{RNAi} lymph glands. Crystal cells are shown in gray. Lamellocytes are rarely found in secondary lobes.
(f, g) Scavenging ROS suppresses differentiation associated with ND75 disruption. Overexpression of Gtpx-1 in *ND75*^{RNAi} lymph glands (in f and g) potently suppresses differentiation into all three lineages as there is a decrease in P1 (red in f), ProPO (gray in g) and L1 (red in g) expression. Compare (f, g) with (d, e). Controls for titration of GAL4 are shown in Supplementary Figure 6.

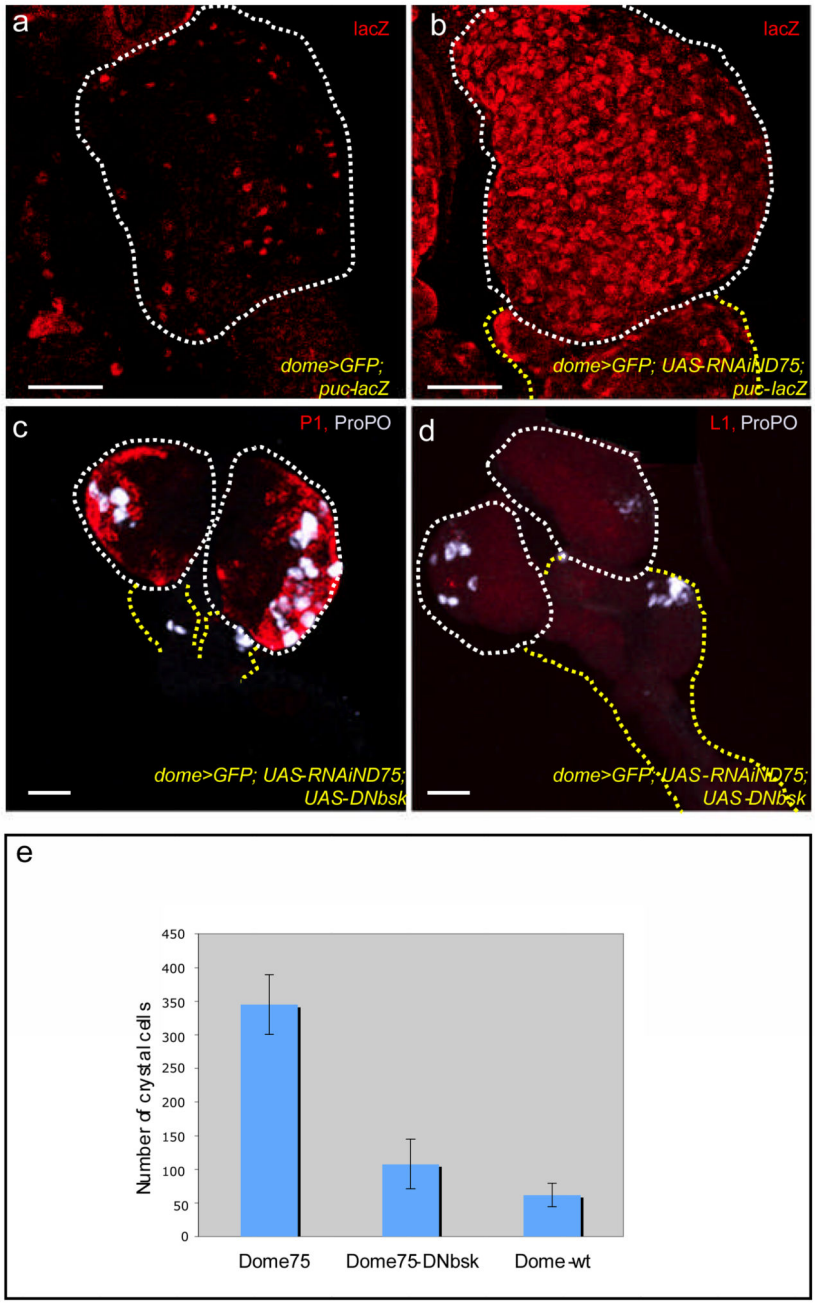

Figure 3. Disrupting JNK signaling suppresses the ROS-dependent differentiation Phenotype

The progenitor population also expresses the MZ marker, *dome-gal4, UAS-2xEYFP* (green), in panels (a–d), but this has been omitted for clarity. Lymph glands that express a RNAi construct to *ND75* (*dome-gal4, UAS-2xEYFP; UAS-RNAiND75)*, are abbreviated as $ND75^{RNAi}$. Scale bars: 50μm.

(a, b) JNK signaling is activated upon ROS increase. *puc-lacZ* expression (red) in WT lymph glands (a) and $ND75^{RNAi}$ lymph glands (b). *puc-lacZ*, which is a transcriptional reporter of JNK signaling is dramatically elevated in $ND75^{RNAi}$ cells.

(c, d) JNK signaling is required for triggering differentiation associated with ND75 disruption.

Expressing a dominant negative construct of JNK in the precursor population ameliorates the effect of complex I disruption as the number of plasmatocytes (red in c) crystal cells (gray in c and d) and lamellocytes (red in d) are reduced virtually to WT levels. Compare (3c, d) with (2d, e)

(e) Suppression of the number of crystal cells formed in $ND75^{RNAi}$*UAS-DNbsk* lymph glands relative to $ND75^{RNAi}$ lymph glands. Error bars are s.e.m and n = 10.

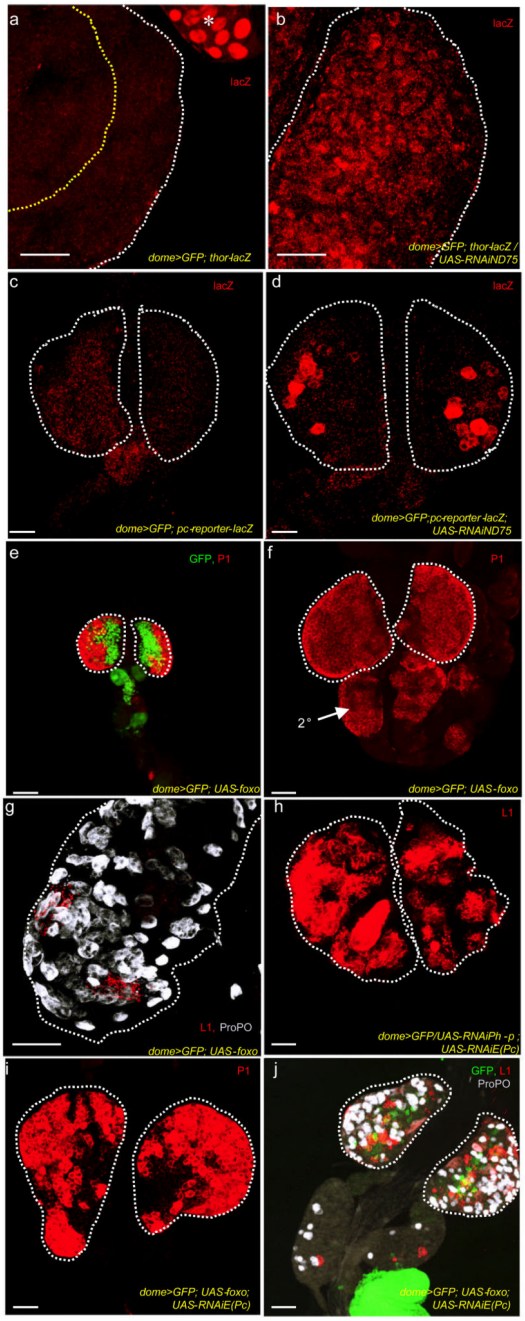

Figure 4. FoxO activation and Polycomb downregulation phenocopy aspects of the ROS induced differentiation

In all panels, the progenitor cells express the MZ marker, *dome-gal4, UAS-2xEYFP* (green), omitted in some panels for clarity. Lymph glands from *dome-gal4, UAS-2xEYFP* larvae were used as wild-type controls (abbreviated WT). Lymph glands which express a RNAi construct to *ND75* in the progenitor cells (*dome-gal4, UAS-2xEYFP; UAS-RNAiND75)*, are abbreviated as $ND75^{RNAi}$. Scale bars :40μm.

(a, b) Disruption of ND75 leads to induction of the FoxO reporter, *thor-lacZ*. WT lymph glands (a) do not express *thor-lacZ* (absence of red). The asterisk in a, points to *thor-lacZ* expression in the ring gland, adjacent to the lymph gland which serves as an internal control. *thor-lacZ* expression is significantly induced in $ND75^{RNAi}$ lymph glands (b).

(c, d) Disruption of ND75 leads to expression of the *polycomb* reporter. The *polycomb* reporter (red) is not expressed in WT lymph glands (c), but is induced in $ND75^{RNAi}$ lymph glands (d).

(e–g) FoxO overexpression causes an increase in plasmatocytes and crystal cells, but has virtually no effect on lamellocytes.

(e) Overexpression of FoxO in the progenitor cells (*dome-gal4, UAS-2xEYFP; UAS-foxo*) causes their premature differentiation into plasmatocytes as shown for earlier than normal P1 staining (red) in a second instar lymph gland. Compare with Figure 2a.

(f) Progenitor cells expressing FoxO in the MZ of the third instar lymph gland also initiate extensive differentiation into plasmatocytes (red). In addition, there is ectopic differentiation in the secondary lobes (arrow, 2°).

(g) FoxO expression in the MZ results in an increase in the number of crystal cells (gray). However, only a few isolated L1-positive cells (red) are evident even in late third instar lymph glands. This image is acquired at twice the magnification of the other panels to highlight the few lamellocytes (red).

(h) RNAi-mediated downregulation of the expression of two polycomb proteins, Enhancer of polycomb, E(Pc) and polyhomeotic proximal (Ph-p), leads to a robust increase in lamellocytes, that stain for L1 (red).

(i, j) When FoxO and the RNAi construct to E(Pc) are expressed together in the MZ progenitors there is an increase in all three mature cell markers.

Co-expression of FoxO and an RNAi construct to E(Pc) trigger the full differentiation phenotype associated with complex I disruption as there is an increase in the number of plasmatocytes (red in i), crystal cells (gray in j) and lamellocytes (red in j).